

WOUND CARE PRODUCTS CONTAINING KERATIN**Field**

5 The invention relates to wound care products containing keratin.

Background of the invention

Wounds and lesions can be caused by a variety of events, including surgery, traumatic injury, burns, abrasions and skin grafts. Healing of wounds may be difficult and may
10 result in problems such as ulcers and septicemia. Of particular concern are chronic wounds, such as pressure sores and diabetic ulcers. The treatment of these conditions is of increasing importance as the population ages. The conventional cascade of biochemical processes which occurs in wound healing, involving hemostasis and inflammation, granulation tissue formation and reepithelization and remodeling, is
15 disrupted in the case of chronic wounds due in part to the prolonged inflammatory response which occurs, and the release of destructive enzymes by inflammatory cells.

It has been recognized for some time that maintaining a moist environment can improve the rate of wound healing. Many products have been developed which provide this
20 environment in order to increase the rate of repair of chronic wounds. The materials used in these dressings are biocompatible to some extent, and include polylactic acid, chitin, alginate derivatives and collagen. The response of these materials to wound exudates, and the biochemical environment that these materials provide are fundamental to their performance in the wound.

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Commercially available dressings include various synthetic materials such as silicone compounds, nylon fabrics, or petrolatum gauzes and the like [A. J. Platt, A. Phipps and K. Judkins, A comparative study of silicone net dressing and paraffin gauze dressing in skin-grafted sites, *Burns*, 22(7), 1996, p. 543-545; Claudia Valenta and Barbara G.
30 Auner, The use of polymers for dermal and transdermal delivery, *European Journal of Pharmaceutics and Biopharmaceutics*, 58(2), 2004, p. 279-289]. Whilst these conventional wound dressing materials are inexpensive and easily available, they generally have poor affinity with the wounded area, insufficient vapour permeability are ultimately unsatisfactory with regards to long term healing of chronic wounds [Marcel

F. Jonkman, Izaäk Molenaar, Paul Nieuwenhuis, Peter Bruin and Albert J. Pennings, New method to assess the water vapour permeance of wound coverings, *Biomaterials*, 9(3), 1988, p. 263-267]. High-performance wound dressing materials are often derived from natural materials having properties similar to those of the patients' skin.

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Wound dressing can be created using natural materials or through combination of synthetic materials and natural materials (JP# 47470/1988; Jen Ming Yang and Hao Tzu Lin, Properties of chitosan containing PP-g-AA-g-NIPAAm bigraft nonwoven fabric for wound dressing, *Journal of Membrane Science*, 243(1-2), 2004, p. 1-7). The use of wound dressings is an extremely important part of wound management and vital to achieve successful healing outcomes [Gordon Freedman, Hyacinth Entero and Harold Brem, Practical treatment of pain in patients with chronic wounds: pathogenesis-guided management, *The American Journal of Surgery*, 188(1), 2004, p. 31-35]. An optimum wound dressing protects the injured tissue, maintains a moist environment, is water permeable, maintains microbial control, delivers healing agents to the wound site, is easy to apply, does not require frequent changes and is non-toxic and non-antigenic.

Currently several forms of wound dressings materials are available commercially, including occlusive dressings, non-adherent dressings, absorbent dressings, and dressings in the form of sheets, foams, powders and gels. Attempts have been made to provide improved dressings, particularly for chronic wounds, that assist in the wound healing process by using biological materials such as cells and growth factors. To date, these biologicals have proven very costly due to factors such as manufacturing processes and storage and stability issues, and in addition they have shown minimal clinical relevance in accelerating the chronic wound healing process. Above all effective, wound management requires an understanding of the process of tissue repair and knowledge of the properties of the wound dressing materials. Only when these two factors are considered together can the process of dressing selection be undertaken in a rational and informed fashion.

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Keratin proteins are present in a wide range of biological tissue, performing a structural role in skin, hair and other materials. Keratins extracted from hair have been shown to

be a valuable component in wound dressings. US 5932552 provides a biocompatible keratin material prepared by either reduction or oxidation for use as a component in wound care products. Those methods included in the art for the oxidation of keratins to create a polar group are harsh and degrading to the keratin, causing protein damage and loss of core physical characteristics arising from the protein amino acid composition and tertiary structure. In addition the oxidation processes used in the preparation of these materials are irreversible and the cysteic acid groups formed cannot be reconverted to cystine to perform a useful structural function. Those methods included in the art for reduction to create soluble proteins are conducted under harsh alkaline conditions that also cause damage to the protein and loss of the core physical characteristics of the keratin proteins.

The core components of keratin fibres, specifically the intermediate filament proteins and the matrix proteins present in wool and hair, play particular roles within the fibre which is reflected in their tertiary structure and amino acid composition. These same features can be capitalized upon to create materials with good physical properties and highly absorbing capacities when using purified forms of these proteins. In order to do this, methods used for isolating keratins need to be mild, to prevent protein damage, create cystine modifications that are reversible, to allow for reconstitution of tough materials through the creation of cystine bonds, and facilitate the isolation of specific keratin protein fractions from the keratin source. The present invention provides new materials for use in wound care products that are prepared according to these principles.

Object of the Invention

It is an object of the invention to provide a wound care product which uses a keratin protein fraction. It is a further object of the invention to provide a keratin protein fraction that is intact and S-sulfonated for use in wound care, or to at least provide the public with a useful choice.

Summary of the Invention

The invention provides a material for treating a wound comprising a keratin protein fraction in which the protein fraction is intact.

The invention also provides a material for treating a wound comprising a keratin protein fraction in which the protein fraction is from the intermediate filament protein family.

The invention also provides a material for treating a wound comprising a keratin protein fraction in which the protein fraction is from the high sulphur protein family.

5 The invention also provides a material for treating a wound comprising a keratin protein fraction in which the protein fraction is s-sulfonated.

The protein fraction may be hydrolysed.

The protein is preferably s-sulfonated.

The protein may be from the high sulphur protein family.

10 The protein may be an intermediate filament protein.

The material is preferably a fibre, a film, a foam or a hydrogel.

The invention also provides a method a method for making a wound care product comprising

a) preparing a 10% solution of a keratin protein;

15 b) mixing the keratin protein and a water soluble polymer to form an intimate mixture;

c) casting the aqueous mixture so produced; and

d) freezing and thawing in sequence to produce a hydrogel.

20 The physico-mechanical properties of the biomaterials may be improved by introducing cross-linker agents to form disulfide bonds and thus remove sulfonate functionalities.

The cross-linking agent used as a reductant may be a thiol or thioglycollate salt.

The physico-mechanical properties may be wet and dry strength.

The thioglycollate salt may be ammonium thioglycollate solution.

25 The water soluble polymer may be polyvinyl alcohol, polyvinylpyrrolidone, polyethylene glycol or the like.

The invention also provides a method of improving the wet strength properties of the wound care products produced by the method of the invention by incorporating a cross-linking agent into them.

The cross-linking agent is preferably an aldehyde.

30 The cross-linking agent may be selected from the group consisting of formaldehyde, glyoxal, glutaraldehyde and the like.

Brief Description of the Drawings

The invention will now be described, by way of example only, with reference to the following specific embodiments:

Figure 1: shows the response of wounds treated with keratin and other materials

5 **Figure 2:** shows sheep fibroblast proliferation on keratin materials;

Figure 3: shows human fibroblast proliferation on keratin materials;

Figure 4: shows the effect of Con(A) stimulation on T-cell growth in the presence of keratin matrices; and

10 **Figure 5:** shows the effect of Con(A) on stimulation of T-cell growth in the presence of keratin matrices after 72 hours.

Detailed Description of the Invention

The hard alpha keratin proteins such as those derived from human hair, wool, animal fibres, horns, hooves or other mammalian sources, can be classified into particular components according to their biochemical properties, specifically their molecular weight and amino acid composition. Table 1 illustrates the amino acid composition determined by conventional analytical methods of typical keratin protein fractions known in the art and also the subject of this invention. This involves acid hydrolysis of the analyte which converts all cystine and labile cystine derivatives to cysteine, typically recorded as half-cystine.

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	SIFP	SHSP	SPEP	IFP	HSP	Whole wool
Cya	0.4	1.7	0.7	0	0	0
Asp	7.9	2.6	8	9.6	2.3	5.9
Glu	15.4	8.6	15	16.9	7.9	11.1
Ser	10.9	14.3	11.4	8.1	13.2	10.8
Gly	8.1	9.1	8.4	5.2	6.2	8.6
His	0.9	0.8	0.9	0.6	0.7	0.8
Arg	7.9	6.8	6.9	7.9	6.2	6.2
Thr	6.5	10.4	6.5	4.8	10.2	6.5
Ala	7.5	3.6	7.5	7.7	2.9	5.2
Pro	5.4	12.6	5.7	3.3	12.6	6.6
Tyr	1.1	1.8	1.2	2.7	2.1	3.8
Val	6.5	6.3	5.8	6.4	5.3	5.7
Met	0.2	0	0.3	0.6	0	0.5
Lan	0.2	0.2	0.3	0	0	0

Ile	3.7	2.9	3.4	3.8	2.6	3
Leu	8.9	3.9	8	10.2	3.4	7.2
Phe	2.5	1.5	2.1	2	1.6	2.5
Lys	2.1	0.4	2.1	4.1	0.6	2.7
Cys	4.2	12.4	4.6	6	22.1	13.1

Table 1: amino acid composition of keratin fractions: S-sulfonated keratin intermediate filament protein (SIFP), S-sulfonated keratin high sulfur protein (SHSP), S-sulfonated keratin peptide (SPEP) as used in the invention. Intermediate filament protein (IFP), high sulfur protein (HSP) and whole wool courtesy of *Gillespie and Marshall, Variability in the proteins of wool and hair, Proc. Sixth Int. Wool Text. Res. Conf., Pretoria, 2, 67-77, 1980*. All residues expressed as mol%. S-sulfocysteine, cystine and cysteine are measured as S-carboxymethyl cysteine following reduction and alkylation, and reported as cys.

Table 2 illustrates the molecular weight determined by conventional analytical methods of typical keratin protein fractions known in the art and also the subject of this invention. Conventional analysis involves cleavage of cystine bonds within the keratin using reduction so that the protein mass is determined in its native, uncrosslinked state, most similar to the unkeratinised state of the protein. Mass is determined using polyacrylamide gel electrophoresis. In the case of the peptide SPEP mass is determined using mass spectrometry. Using these methods the keratin is made soluble without any hydrolysis of peptide bonds and an accurate measure of molecular weight is determined.

Keratin protein fraction	Molecular weight/kD
SIFP	45-60
SHSP	10-25
SPEP	<1
IFP	45-60
HSP	11-23

Table 2: Molecular weight of keratin fractions: S-sulfonated keratin intermediate filament protein (SIFP), S-sulfonated keratin high sulfur protein (SHSP), S-sulfonated

keratin peptide (SPEP) as used in the invention. Intermediate filament protein (IFP) and high sulfur protein (HSP) courtesy of Gillespie and Marshall, *Variability in the proteins of wool and hair*, *Proc. Sixth Int. Wool Text. Res. Conf., Pretoria, 2*, 67-77, 1980.

5 Both amino acid composition and molecular weight varies, to a small extent, across keratin types, between species and also within breeds of one species, for example between wools from different breeds of sheep. The figures given in Tables 1 and 2 are indicative for the keratin source stated. However, individual types of keratin proteins, or keratin protein fractions, have distinctive characteristics, particularly molecular
10 weight and amino acid content.

The subject of the invention is materials containing intact S-sulfonated keratin protein fractions. "Intact" refers to proteins that have not been significantly hydrolysed, with hydrolysis being defined as the cleavage of bonds through the addition of water.
15 Gillespie (Biochemistry and physiology of the skin, vol 1, Ed. Goldsmith Oxford University Press, London, 1983, pp475-510) considers "intact" to refer to proteins in the keratinized polymeric state and further refers to polypeptide subunits which complex to form intact keratins in wool and hair. For the purpose of this invention "intact" refers to the polypeptide subunits described by Gillespie. These are equivalent to the keratin
20 proteins in their native form without the disulfide crosslinks formed through the process of keratinisation.

Keratin protein fractions are distinct groups from within the keratin protein family, such as the intermediate filament proteins, the high sulfur proteins or the high glycine-
25 tyrosine proteins well known in the art. Intermediate filament proteins are described in detail by Orwin et al (*Structure and Biochemistry of Mammalian Hard Keratin*, *Electron Microscopy Reviews*, 4, 47, 1991) and also referred to as low sulphur proteins by Gillespie (Biochemistry and physiology of the skin, vol 1, Ed. Goldsmith Oxford University Press, London, 1983, pp475-510). Key characteristics of this protein family
30 are molecular weight in the range 40 – 60 kD and a cysteine content (measured as half cystine) of around 4%. The high sulfur protein family are also well described by Orwin and Gillespie in the same publications. This protein family has a large degree of

heterogeneity but can be characterised as having a molecular weight in the range 10 – 30 kD and a cysteine content of greater than 10%. The subset of this family, the ultra high sulfur proteins can have a cysteine content of up to 34%. The high glycine-tyrosine protein family are also well described by Orwin and Gillespie in the same publications.

5 This family is also referred to as the high tyrosine proteins and has characteristics of a molecular weight less than 10 kD, a tyrosine content typically greater than 10% and a glycine content typically greater than 20%.

For the purpose of this invention a “keratin protein fraction” is a purified form of
10 keratin that contains predominantly, although not entirely, one distinct protein group as described above. In the context of this invention S-Sulfonated keratins have cysteine/cystine present predominantly in the form S-sulfocysteine, commonly known as the Bunte salt. This highly polar group imparts a degree of solubility to proteins. Whilst being stable in solution, the S-sulfo group is a labile cysteine derivative, highly
15 reactive towards thiols, such as cysteine, and other reducing agents. Reaction with reducing agents leads to conversion of the S-sulfo cysteine group back to cysteine. S-sulfo cysteine is chemically different to cysteic acid, although both groups contain the SO_3^- group. Cysteic acid is produced irreversibly by the oxidation of cysteine or cystine and once formed cannot form disulfide crosslinks back to cysteine. S-sulfocysteine is
20 reactive towards cysteine and readily forms disulfide crosslinks.

SIFP can be prepared by methods such as those described in WO03011894.

The aspect and other details of the invention will now be more particularly described.

25 Highly S-sulfonated keratins have been shown to be able to be formed into a variety of matrices including porous sponges, films and fibres using methods such as those outlined in NZ/PCT/00169 (which is incorporated herein).

Purified wool keratin intermediate filament proteins are particularly well suited to
30 reformation into matrices, due in part to their high molecular weight and their tertiary structure. Methods outlined in NZ/PCT/00169 make extensive use of these materials to form useful matrices.

S-sulfo keratins can be prepared by a variety of methods, including those outlined in PCT/NZ02/00125 (which is incorporated herein).

5 Porous sponge matrices are of particular use in a wound environment as they can play an important role in absorbing wound exudates and maintaining a healthy environment for healing a wound. In addition they can act as media for the delivery of other healing agents, such as growth factors, antibacterial agents or cultured cells, that stimulate the healing process. These features are enhanced through using S-sulfonated keratin protein
10 fractions to construct the matrices. The highly polar nature of the S-sulfo group makes matrices derived from this material highly absorbing. In addition, S-sulfonated keratins are biocompatible and do not invoke an adverse response in vitro.

Films are an important component in dressings for the treatment of wounds, providing a
15 barrier to protect the wound and maintaining an appropriate environment to encourage healing. S-sulfonated keratins films are biocompatible and do not invoke an adverse response in vitro. As such, they are useful components in a wound dressing.

Fibres reconstituted from S-sulfonated keratin intermediate filament proteins can be
20 used as a component for wound dressings. Fibres are particularly versatile as they can be formed into woven or non-woven constructs and fibre design can be used as well control of the chemistry of the material to effect the interaction of the dressing with the wound. Much work has been undertaken into the use of regenerated fibres in wound care, in particular alginate fibres. Reconstituted keratin fibres derived from S-
25 sulfonated intermediate filament proteins are a new material for use in similar applications.

Hydrogels are frequently used in wound dressings and play an in important role in controlling the wound environment and provide a suitable medium for the delivery of
30 actives to stimulate or assist healing. S-sulfonated keratins, in particular S-sulfonated intact keratin intermediate filament protein, is an excellent substrate for the formation of

hydrogels as a result of the high degree of order and intermolecular interaction achievable as a result of the intact nature of the proteins.

Keratin materials derived from the SIFP and SHSP protein fractions contain differing amounts of the highly polar S-sulfo group, and consequently differ in their physicochemical characteristics, in particular their ability to absorb moisture. Wound dressings derived from a combination of these absorb moisture to a greater or lesser extent, and so can be controlled in the degree to which they will absorb wound exudates.

S-sulfonated keratin proteins prepared as spray or freeze dried powders are highly absorbing materials that are a valuable component in wound dressings, in particular for use in the hydrogel type dressing in which alginates or collagen derivatives are the materials used frequently in currently available products. Combination of the SIFP and SHSP proteins leads to a degree of control over the absorbing capacity of the powder and the nature of the gel formed on absorbance, due to the variation in the amount of S-sulfo groups present within each protein fraction.

Due to the intact nature of the proteins, and the water solubility of the material arising from the presence of the polar S-sulfonate group, S-sulfonated keratin protein fractions, in particular the keratin intermediate filament protein fraction, can be readily formed into a variety of matrices and the physical properties of these matrices are such that they can provide a useful physical role in a wound environment. Furthermore, the materials can be chemically treated following reformation into films, fibres or sponges, to remove the S-sulfonate functionality and generate disulfide crosslinks within the material, similar to those present in the native keratin. Methods for this treatment are described in NZ/PCT/00169. When treated in this way, the keratin matrices are less absorbing and retain their structure in a wound environment. They are well suited to the delivery of bioactives to the wound site, such as antibacterial agents, growth factors, antibiotic treatments, cultured cells or other drugs.

The physical and mechanical properties of the wound dressing or healing membranes can be readily improved through a variety of methods. One method involves treatment with a reducing reagent such as ammonium thioglycolate solution at pH = 7.0 for 1 hour in order to remove the sulfonate functionality from S-sulfonate keratin and introduce cystine disulfides as crosslinks. This causes significant improvement in the mechanical properties particularly wet strength of the membranes materials. Chemical conversion is confirmed using Fourier-Transform Infra-Red (FT-IR) spectroscopic studies as the S-sulfonated group gives rise to a strong and sharp absorbance at 1022 cm⁻¹ which is observed to disappear on exposure of the S-sulfonated to the reagents described.

A method for the improvement of the physical and mechanical strength of keratin hydrogel biomaterials is to increase the hydrogen bonding network between the keratin protein chains or the keratin protein and other polymers, such as polyvinyl alcohol and polyvinyl pyrrolidone. This can be achieved by using a freezing-thawing process during the constructing hydrogel sheets. This is confirmed by an increase in the insolubility of hydrogels formed using this process.

Production of a chemically cross-linked hydrogel biomaterial is another embodiment in the invention. Improving physical properties such as insolubility and strength in swollen states can be achieved by using chemical cross-linkers such as glutaraldehyde, that allow the formation of chemical cross-links between keratin protein chains.

Further, the physical properties of the keratin based membranes and hydrogels can be increased by standard protein cross-linking methods including using, typical chemical cross-linkers such as, glutaraldehyde, formaldehyde, carbodiimides, e.g., 1-ethyl-3-(dimethylaminopropyl)carbodiimide, 2,5-hexanedione, diimidates, e.g., dimethylsuberimide, or bisacrylamides, e.g., N,N'-methylenebisacrylamide.

In vitro testing

The biological response for the materials described above has been determined *in vitro* through growth of cells relevant in wound healing, and immunogenic response, specifically fibroblasts and lymphocytes.

Keratin material	Cystine functionality	Label
Sponge	S-sulfonate	A
Sponge	disulfide	B
Film	S-sulfonate	C
Film	disulfide	D
Powder	S-sulfonate	E

Table 3: Keratin materials tested in vitro.

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Sheep Fibroblasts:

Figure 2 is a graph showing the effect of different keratin matrices on ovine dermal fibroblast cell proliferation relative to cell media alone (control). Parallel samples of n
 10 = 3 were used for each time point.

The proliferation kinetics of ovine fibroblasts on most of the keratin matrices is similar. Wells are initially seeded with ~10,000 cells (0 hours). During the first 24h post-seeding, the culture experiences a lag time as evidenced by the decline in cell numbers.
 15 This phenomenon has been recognised in all assays performed and the drop is observed in control wells in addition to those containing the test materials. Additional shorter time-course experimentation has shown that this lag time lasts for less than 12h (data not shown) and that the exponential phase of growth begins at this point. Population doublings occur approximately every 24h-48h with subconfluency (approximately 80%
 20 confluency) marking the end of logarithmic growth. This corresponds to the end of the experimental time course (5 days or 120h). Extended time-course experiments have indicated a plateau in cell growth shortly after this with full confluence of the culture. Contact inhibition and depletion of nutrients play a key role in limiting the growth rate at this point and the monolayer culture exhibits signs of cell death (i.e. loss of
 25 membrane integrity, reduction in cell numbers, vacuolisation of individual cells).

Such kinetics are exhibited by sheep fibroblasts on most of the biopolymer substrates, particularly the films and sponge samples.

With respect to the individual matrix types, the following observations were made:

Films. The films with the disulfide chemical configuration support sheep fibroblast growth most satisfactorily (material D). A second sodium S-sulfonate salt configuration demonstrated by film material C supports cell growth to a lesser degree and tends to swell in culture. Cells on these films showed typical multi- or bipolar elongated fibroblastic morphology with good spread.

Sponge. Fibroblast growth porous sponge material B (disulfide configuration) matched that of some of the better films. During the assay, cells were witnessed to attach to the upper surface of the sponge. By light microscopy, the morphological appearance of these cells was deemed similar on all substrates compared to the no-matrix control. Cells were observed by microscopy to infiltrate the sponge material.

Powder. A keratin powder dilution series was established and the result presented in the graph as material E. This result represents the observed growth curve for the concentration 2mgml^{-1} . Higher concentration solutions than this resulted in the same curve, lower demonstrated a slightly higher cell proliferation rate than the control.

Extract tests suggest the keratin powder itself may be, at sufficient concentration, mitogenic for sheep fibroblasts.

Human Fibroblasts:

Figure 3 is a graph showing the effect of different keratin matrices on human dermal fibroblast cell proliferation relative to cell media alone (control)

Human fibroblastic data for the corresponding keratin substrates more or less mirrored that observed with the sheep cell line. Again a typical growth curve was established over the 120h period, however 100% confluence was reached in the control wells by the end of this time. At 120h, cultures grown in the presence of the majority of test materials ranged from 83-89% confluence.

Sheep Lymphocytes

Figure 4 demonstrates the effect of ConA stimulation on T cells grown in the presence/absence of keratin matrices over a 10-day period. Tritiated thymidine counts were converted to cell numbers per well (against a series of standards) for each of the treatment groups.

Resulting analysis suggests:

1. There is a marked difference in cell numbers over the 240h experiment between ConA stimulated and non-activated sheep T lymphocyte cells. Control (grown in the absence of keratin biopolymers) cell numbers show a 6-fold difference between unstimulated and stimulated cells at 240h. Cells grown in the absence of conA reached concentrations of 50000 cells/well at Day 10, whilst control cells with ConA supplementation exceeded 300000 cells/well at the same point. Such high concentrations were obtainable as the cells were maintained in suspension culture therefore reduced nutrient supply and not surface area requirement was the limiting factor.

2. There was little difference in cell proliferation rates between sample (matrix presence) and control (matrix absence) wells. This effect was noted for both stimulated cells and unstimulated cells. In other words:

- (a) Unstimulated cells grown in the presence of matrices proliferated at the same degree as those grown purely on tissue culture reference wells. This indicates that although the keratin biomaterials are not non-immunogenic, they look to be antigenically inert. If they were non-immunogenic, one would expect no proliferation of lymphocytes exposed to the biomaterial. If indeed it is inert, cell proliferation rates would mimic those of the control as appears the case.

- (b) Stimulated cells grown in the presence of the matrices proliferated at a similar rate or slightly higher than the control wells (which contained no

keratin matrix). This suggests the activated T-cells are not being inhibited in any way by the matrix itself or any degradative by-products it may produce over this short time-course. Failure to inhibit active T-cells by the tested biomaterials demonstrates the product does not interfere with the normal cell-mediated immune response.

Figure 5 shows the effect of ConA stimulation on T-lymphocytes cells grown in the presence of a variety of matrices at 72h. Total counts reflect the level of thymidine uptake and incorporation into DNA, which is then used as a measure of proliferation (see in the previous graph). A 72h culture is regarded as the best measure of time for comparison between treatments as the cells are well within exponential phase growth.

The results are presented as a vertical bar graph with stimulated and non-stimulated treatments beside each other. Error bars represent means \pm SD for $n = 3$. The unstimulated well counts (unlabelled) show very little variation with small error scores. Total counts for stimulated cells are slightly more variable although student T test analyses indicate only the material C is significantly different ($p = 0.075$) from the control.

Unprimed T cells were shown to proliferate at the same degree in the presence or absence of the matrices. This showed the biomaterials were not non-immunogenic but instead inert. No single matrix tested stimulated the normal immune response to any degree greater than the control (no matrix well series).

Activated T cells maintained in culture with keratin matrices proliferated normally at a similar or greater than rate compared to those of the control (no matrix present). This demonstrates the biomaterials are biocompatible with stimulated T cells, mitogenic to a degree and most certainly do not interfere with the normal immune response. There was no inhibition of activated T cells by any matrix or its byproducts.

In summary, the tested matrices do not interfere with the body's cell-mediated immune response and are biocompatible with a sheep T lymphocyte cell line.

In vivo testing

The effect of keratin matrices in a wound environment was determined using an animal model.

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A randomised trial was conducted applying 4 samples to groups of rats with excision wounds. There were 6 male rats per group. Two wounds (8 mm diameter) were established on the back of each rat along the mid-line. One wound served as the control with the vehicle or saline was applied and the test material was applied to the other wound. The rate of healing was monitored by regular photographs. The wounds were photographed every second day and the area of the healing wounds were quantified. The percentage (%) change for each wound was determined at each time point and relative rate of healing of the experimental to the control wound determined for each rat. The mean difference at each time was then calculated and is detailed in table 4.

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The dressings studied were: **KP-U**: Keratin membrane wound dressing (example 1), **KP-T**: Keratin membrane wound dressing (example 3), **HG-GL**: Keratin hydrogel (example 4), **HG-O**: Keratin hydrogel (example 2), **HG-C** commercially available hydrogel wound dressing product.

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Twenty-four rats were used in the trial and trial studies were carried up to healing endpoint.

Results based on wound healing rate can be summarized as follows:

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- i) the **HG-GL** wound dressing material significantly hastened the healing, with the most marked difference occurring in the early stages of healing.
- ii) the **KP-T** wound dressing material showed some improvement in healing rate, especially over the first 3 to 5 days.

Days after wound administration	Wound area, % of original					
	KP-U	KP-T	HG-O	HG-GL	HG-C	Blank
1	100	100	100	100	100	100
3	102	90	97	77	97	93

5	94	90	81	70	90	84
9	64	69	-	-	-	-
11	39	33	35	24	40	36
13	28	24	23	13	25	23
15	15	11	19	9	13	18
17	18	11	14	5	6	12
19	5	3	8	2	2	6

Table 4: Area occupied by wound site following administration of various wound dressings. **KP-U:** Keratin membrane wound dressing (example 1), **KP-T:** Keratin membrane wound dressing (example 3), **HG-GL:** Keratin hydrogel (example 4), **HG-O:** Keratin hydrogel (example 2), **HG-C** commercially available hydrogel wound dressing product.

Examples

The methods for the preparation of various forms of keratin are now described by way of example.

Example 1: PRODUCTION OF KERATIN MEMBRANES FOR USE IN A WOUND CARE PRODUCT

A 10% S-sulfonated keratin intermediate filament protein (SIFP) solution was prepared using of S-sulfonated keratin intermediate filament protein powder dissolved in distilled water with gradual addition of 1M NaOH over 2 hours under mechanical stirring. The pH was maintained in the range 8.0–9.5, and finally adjusted to 8.5. The keratin protein solution was centrifuged at 27000 g for 10 mins in order to remove any air bubbles and undissolved material. The resulting keratin protein solution was cast into a petri dish and the solvents evaporated under ambient conditions to leave a keratin membrane. The solvent can also include some percentage of organic based aqueous miscible solvent, such as an alcohol.

Example 2: PRODUCTION OF A KERATIN HYDROGEL FOR USE IN WOUND CARE PRODUCTS

A 10% S-sulfonated keratin intermediate filament protein (SIFP) solution was prepared as describe in **Example 1**. The solution was then intimately mixed with water soluble polymers such as, polyvinyl alcohol (PVA) comprising 20% solid content and polyvinyl pyrrolidone (PVP) comprising 10% solid content to a achieve a optimum rheology and optimal composition i.e., SIFP: PVA: PVP = 100: 60: 40 (w/w, %) for creating hydrogel. The combined solution was then cast, and hardened through a freezing-thawing cycle to produce a keratin based hydrogel. This involved freezing the material at -80°C for 1 hr and thawing at 23°C for 1 hour. This freeze-thaw cycle was repeated up to 7 times to obtain a hydrogel. The resulting hydrogel was washed with distilled water multiple times to remove any unreacted keratin and polymers.

Example 3: PRODUCTION OF CROSS-LINKED KERATIN MEMBRANES FOR USE IN A WOUND CARE PRODUCT

In order to improve the physical strength and also mechanical properties of materials produced as described in **Example 1** membranes were treated with reductants to induce chemical cross-linking. Immersion of the membranes in a solution of 0.25M ammonium thioglycollate adjusted to pH 7.0 for 60 minutes was used to remove the sulfonate group from the S-sulfonated keratin protein (SIFP), and allow the formation of disulfide bonds (-S-S-). The resulting membranes were washed multiple times with distilled water to remove any residual reagents.

Example 4: PRODUCTION OF CHEMICAL CROSS-LINKED KERATIN HYDROGEL FOR USE IN A WOUND CARE PRODUCT

Following preparation of the keratin, PVA, PVP solution as described in **example 2**, 0.05% to 0.1% of glutaraldehyde cross-linking agent was added into the blended solution. The combined solution was then cast, and hardened through a freezing-thawing cycle to produce a keratin based hydrogel. This involved freezing the material

at -80°C for 1 hr and thawing at 23°C for 1 hour. This freeze-thaw cycle was repeated up to 7 times to obtain a hydrogel. The resulting hydrogel was washed with distilled water multiple times to remove any unreacted keratin and polymers, as well residues of unreacted glutaraldehyde. Physical observation showed significant improvement of their dimension stability or strength. These characteristics are also confirmed by the hydrogels insolubility behaviour in aqueous solvent, after having been made as an intimate blending keratin protein and polymers from an aqueous solvent (i.e., water).

Example 5: PRODUCTION OF DISULFIDE CROSS-LINKED KERATIN MEMBRANE FOR USE IN A WOUND CARE PRODUCT

A 10% S-sulfonated keratin intermediate filament protein (SIFP) solution was prepared as describe in **Example 1**. The solution was then intimately mixed with 1% of 0.25M ammonium thioglycollate solution (NH₄TG), where compositions are as: SIFP: NH₄TG = 99 : 1 (w/w, %). The blended solution was then cast into a petri dish and the solvents evaporated under ambient conditions to create a disulfide cross-linked keratin membrane. The resulting membrane was washed with distilled water multiple times to remove any residual NH₄TG.

Example 6: PRODUCTION OF DISULFIDE CROSS-LINKED KERATIN HYDROGEL FOR USE IN A WOUND CARE PRODUCT

A 10% S-sulfonated keratin intermediate filament protein (SIFP) solution was prepared as describe in **Example 1**. The solution was then intimately mixed with 1% of 0.25M ammonium thioglycollate solution (NH₄TG), where compositions were: SIFP: NH₄TG = 99 : 1 (w/w, %). The blended solution was then cast, and hardened through a freezing-thawing cycle to produce a disulfide cross-linked keratin hydrogel. This involved freezing the material at -80°C for 1 hour and thawing at 23°C for 1 hour. This freeze-thaw cycle was repeated up to 7 times to obtain a chemical cross-linked hydrogel.

Example 7: PRODUCTION OF AN UNCROSSLINKED KERATIN HYDROGEL FOR USE IN A WOUND CARE PRODUCT

A 10% S-sulfonated keratin intermediate filament protein (SIFP) solution was prepared as describe in **Example 1**. The solution was cast, and hardened through a freezing-thawing cycle to produce a keratin based hydrogel. This involved freezing the material
5 at -80°C for 1 hour and thawing at 23°C for 1 hour. This freeze-thaw cycle was repeated up to 7 times to obtain a keratin protein hydrogel.

Industrial Applicability

The invention will be useful in a wide range of wound care products. Such products will
10 assist in the healing and rate of healing of wounds by providing a biochemical environment around the wound site that induces healing.